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The Effect of Acid and Alkali Unfolding and Subsequent Refolding on the Pro-oxidative Activity of Trout Hemoglobin

HORDUR G. KRISTINSSON*,† AND HERBERT O. HULTIN‡

Laboratory of Aquatic Food Biomolecular Research, Aquatic Food Products Program, Department of Food Science and Human Nutrition, University of Florida, Gainesville, Florida 32611, and Massachusetts Agricultural Experiment Station, Department of Food Science, University of Massachusetts/Amherst, Marine Station, Gloucester, Massachusetts 01930

The pro-oxidative activity of trout hemoglobin was significantly increased at low pH (2.5−3.5) in a washed fish muscle (WFM) system. It was found that the more unfolded the hemoglobin was the more exposed its heme group was, which increased its pro-oxidative activity. The amount of oxidation products produced (TBARS) were, however, lower at low pH vs neutral pH. At pH 10.5−11, the pro-oxidative activity of hemoglobin was greatly suppressed. The conformation of hemoglobin was significantly more stable at high pH as compared to pH 7 as judged by its visible absorption spectrum. Hemoglobin readjusted from low pH to pH 7 had a higher pro-oxidative activity (i.e., more rapid oxidation) in WFM than native hemoglobin at pH 7, even though TBARS values were lower than in the untreated sample at pH 7. The results suggest that the WFM becomes slightly more susceptible to oxidation after low pH treatment but also produces less TBARS. The increased pro-oxidative activity after pH readjustment correlated well with an incomplete recovery in the native structure on pH readjustment. A longer unfolding time and a lower pH led to a less refolded hemoglobin with increased pro-oxidative activity. Hemoglobin was less pro-oxidative at low pH in the presence of 500 mM NaCl. The presence of salt did, however, increase the pro-oxidative properties of hemoglobin after readjustment to pH 7. The treatment of washed fish muscle at alkaline pH followed by adjustment to pH 7 led to a slight delay in hemoglobin-mediated lipid oxidation in WFM as compared to native hemoglobin at pH 7. The results suggest that WFM becomes less susceptible toward oxidation after pH readjustment from alkaline pH. These results clearly show that for muscle protein extraction/isolation processes requiring highly alkaline or acidic conditions, alkaline conditions are preferred if the lipid oxidation originating from hemoglobin is to be minimized.

KEYWORDS: Hemoglobin; lipid oxidation; trout; low pH; high pH; molten globule; conformation; unfolding; refolding

INTRODUCTION

The presence of blood in fish muscle is known to lead to oxidation and color problems (*1*, *2*). The main protein component of blood is hemoglobin, which is highly concentrated in the erythrocytes at ~330 mg/mL (*3*). Fish hemoglobins have received little attention in the past in terms of their role in fish lipid oxidation. However, recent studies have shown that they can be very potent pro-oxidants (*2*, *4*−*6*) and are the principal pro-oxidants under some conditions. Hemoglobin can be present in many different forms (*7*). In its reduced state, the iron can be bound to O_2, which is stabilized via hydrogen bonding by the nearby distal histidine (*8*), or it can be without the oxygen, such as at low pH (*8*) or at low oxygen tension (*9*). The iron can oxidize to form the brown methemoglobin under the right solution conditions. Both the oxidized and the reduced forms can be pro-oxidative. Several different mechanisms of the pro-oxidative power of hemoglobin have been proposed (*7*). Oxyhemoglobin (Fe^{2+}−O_2) can autoxidize to methemoglobin (Fe^{3+}) releasing its oxygen as a superoxide anion radical ($O_2^{\bullet -}$). This radical can further dismutate to hydrogen peroxide, which can activate the methemoglobin to form a hypervalent ferryl hemoglobin ($Fe^{4+}{=}O$). Although this species is only transient in nature and has a short half-life, it is capable of peroxidizing lipids and is thought to be the main species responsible for hemoglobin-induced lipid oxidation in muscle products (*10*). This ferryl species has a protein radical form and exerts its action by abstracting an electron from the lipid substrate leaving a free radical substrate species, which can cause further oxidation. The superoxide released on autoxidation can also lead to the formation of species ($HOO^\bullet$ and $HO^\bullet$) that are pro-oxidative,

* To whom correspondence should be addressed. Tel: 352 392-1991. Fax: 352 392-9437. E-mail: hgkristinsson@mail.ifas.ufl.edu.
† University of Florida.
‡ University of Massachusetts/Amherst.

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HO• more so than HOO•. In a second mechanism, which is poorly understood, the protein may act as a pseudo-lipoxygenase (*7*).

Little is known how highly acidic and alkaline conditions influence the pro-oxidative properties of hemoglobin. This is of significant interest to a recently developed process aimed at recovering fish muscle proteins from underutilized fish species and byproducts, many of which are rich in heme proteins. This process involves extracting fish proteins using a high (∼10.5−11.5) or low (∼2.5−3.5) pH to solubilize the muscle proteins and selectively recovering the soluble proteins after centrifugation by isoelectric precipitation (pH 5.5). During the low and high pH solubilization step, hemoglobin would have an opportunity to oxidize the lipids of fish muscle. Furthermore, hemoglobin may coprecipitate with the muscle protein during isoelectric precipitation and could oxidize the lipids present in the final protein product, which also may result in adverse color changes. The pH can have a great impact on the function of hemoglobin and its pathways as a pro-oxidant. The autoxidation reaction is enhanced by a low pH while it is reduced at an alkaline pH as interactions with distal histidine become stronger (*8*, *11*). Part of this enhancement of autoxidation at a low pH comes from the increased dissociation of the tetramer to dimers for mammalian hemoglobins (*12*) and possibly full dissociation of fish hemoglobin to monomers (*13*). This high sensitivity of some fish hemoglobin components for protons is believed to be due to the presence of positive charges at the subunit interfaces, which on protonation cause a dramatic shift in the spatial arrangement of the subunits (*14*). Dissociation is also accomplished as the protein is diluted, e.g., on erythrocyte lysis (*15*). The dissociated form is also more pro-oxidative (*16*) and has an increased tendency to lose its heme (*17*). The presence of preformed lipid hydroperoxides and other oxidation products may also cause an increase in the autoxidation of hemoglobin (*7*, *18*). Deoxygenation is furthermore favored at low pH, and evidence suggests that this species may play an important role in oxidation as the pH is lowered (*4*). Formation of the reactive HOO• and very reactive HO• from superoxide is favored as the pH is lowered. The former can easily penetrate into and through lipid bilayers while HO• cannot due to its high reactivity. Both HOO• and HO• can participate in lipid oxidation (*19*). The stability of the heme in the protein is also compromised as the pH is lowered likely due to opening of the heme crevice (*20*), which may lead to increased exposure of the heme to fatty acids or its partition into membrane bilayers (*21*, *22*).

The goal of the work presented here was to investigate how the pro-oxidative properties of trout hemoglobin are affected at low and high pH and after readjustment to neutrality in a washed cod muscle system. This work will aid in our understanding on how to minimize lipid oxidation in processes requiring the exposure of a mixture of muscle proteins and hemoglobin to high or low pH.

MATERIALS AND METHODS

Preparation of Trout Hemolysate/Hemoglobin. Rainbow trout (*Onchyrynchus mykiss*) were purchased from Mohawk Trout hatchery (Sunderland, MA) and kept alive in a recirculating water bath (5 °C). The blood was drawn from the caudal vein of trout anesthetized in aminobenzoic acid as described by Rowley (*23*). The hemolysate was prepared from the blood according to Fyhn et al. (*24*) as modified by Richards and Hultin (*4*), where red blood cells were washed and lysed to release hemoglobin. For all studies, the hemoglobin levels were quantified spectrophotometrically as described by Hudzik (*25*). The hemoglobin solutions were kept frozen at −80 °C and were used within 2 months. This did not affect the conformation of hemoglobin as assessed by circular diochroism and UV−visible spectra.

Lipid Oxidation Studies. A batch of washed cod (*Gadus morhua*) muscle matrix was prepared according to Richards and Hultin (*4*) as a membrane lipid substrate. The moisture content of the washed cod was determined using a Cenco moisture balance (CSC Scientific Company Inc., Fairfax, VA). The water was adjusted to 87%, and the washed muscle was kept frozen at −80 °C until needed for the lipid oxidation tests. The following lipid oxidation experiments were performed.

Pro-oxidative Activity of Hemoglobin at Low and High pH. The washed cod matrix was adjusted to either low pH (pH 2.5, 3, and 3.5) or high pH (pH 10.5 and 11) using 1 M HCl or NaOH. A control sample of pH 7 was also prepared. The pH of the systems was checked and verified by suspending a 1 g sample in 10 mL of distilled deionized H_2O. The washed cod matrix was split into two groups: one with no added salt and the other with 500 mM NaCl added. The hemoglobin was added to the matrix and mixed with a plastic spatula to give a concentration of ∼5.8 μmol/kg substrate. This system was kept at 5 °C in Petri dishes with the lid on, and samples were periodically taken and measured for thiobarbituric acid reactive substances (TBARS) according to Lemon (*26*) as modified by Richards and Hultin (*4*).

Changes in the hemoglobin conformation were analyzed at the pH values tested for oxidation using UV−visible spectroscopy. For these tests, the hemoglobin (∼0.8 μM) was added to distilled deionized H_2O at the pH values presented above with or without 500 mM NaCl present and stored at 5 °C. At select time points, changes in the environment of heme, ligand binding, and oxidation state were determined by UV−visible spectroscopy by scanning the hemoglobin solutions from 350 to 700 nm in a Hitachi-3110 double beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA) at 120 nm/min with buffer as a reference.

Pro-oxidative Activity of Hemoglobin Readjusted (Refolded) from Low and High pH. The washed cod matrix was adjusted to pH 2.5, 3.0, and 10.5 with 1 M HCl or NaOH, and the hemoglobin was added to the system as described above. Two unfolding periods were selected as follows: 90 s ("short" unfolding) and 20 min ("long" unfolding). The period of 90 s was chosen since this was the minimum time it took to distribute the hemoglobin uniformly in the washed cod. After being at 90 s or 20 min at the pH above, the system was adjusted to pH 7 with 1 M HCl or NaOH, and the pH was verified as described above. The systems were then transferred into Petri dishes and kept at 5 °C, and samples were taken immediately after readjustment and then periodically and analyzed for TBARS.

Changes in the hemoglobin conformation after pH readjustment from low or high pH were determined by subjecting a hemoglobin solution (∼0.8 μM) to pH 2.5, 3, and 10.5 for 90 s and 20 min followed by pH readjustment to pH 7 with HCl or NaOH. Changes in the conformation were analyzed as described above.

Role of the Washed Cod Muscle Matrix in Oxidation. Two experiments were performed to investigate if changes in the matrix upon acidic/alkaline treatment may be responsible for the changes seen in lipid oxidation. In the first experiment, the hemoglobin solutions were adjusted to pH 2.5 for 90 s or 20 min and to pH 10.5 for 20 min and then readjusted to pH 7 and added to an untreated washed cod matrix. In the second experiment, the washed cod was adjusted to pH 2.5 or 10.5 for 20 min and then readjusted to pH 7, and untreated hemoglobin was added. In both experiments, the systems were kept at 5 °C and analyzed for TBARS periodically. These results were compared to the experiments performed where hemoglobin and washed cod were both treated to low and high pH (see sections above).

Statistical Analysis. All experiments and analyses were conducted in triplicate, and results are reported as means with standard deviations. Analysis of variance was used to determine the significant differences between the samples using the Statistical Analysis System (SAS) computer program (SAS Institute, NC).

RESULTS

Pro-oxidative Activity at Low and High pH. As hemoglobin is a very potent pro-oxidant in muscle-based food systems, it is important to determine how extreme pH treatment would affect its pro-oxidative activity. Initially, it was of interest to study how pro-oxidative hemoglobin was when kept at low and

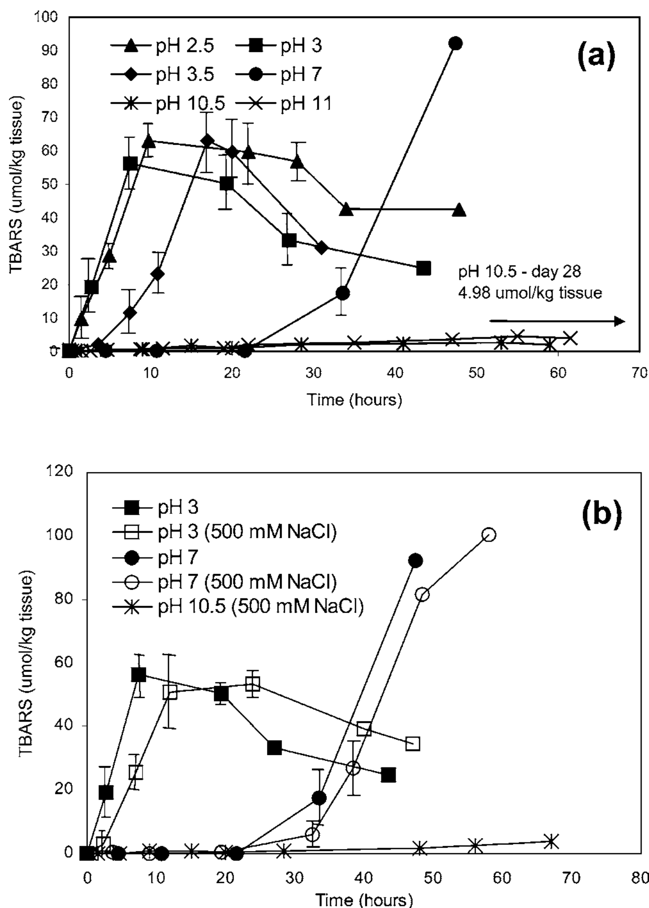

Figure 1. Oxidation of washed cod muscle by trout hemoglobin at different pH values in the absence (**a**) or presence (**b**) of 500 mM NaCl. Hemoglobin was added at 5.8 μmol/kg substrate. The water content was ∼87%. The temperature was 5 °C.

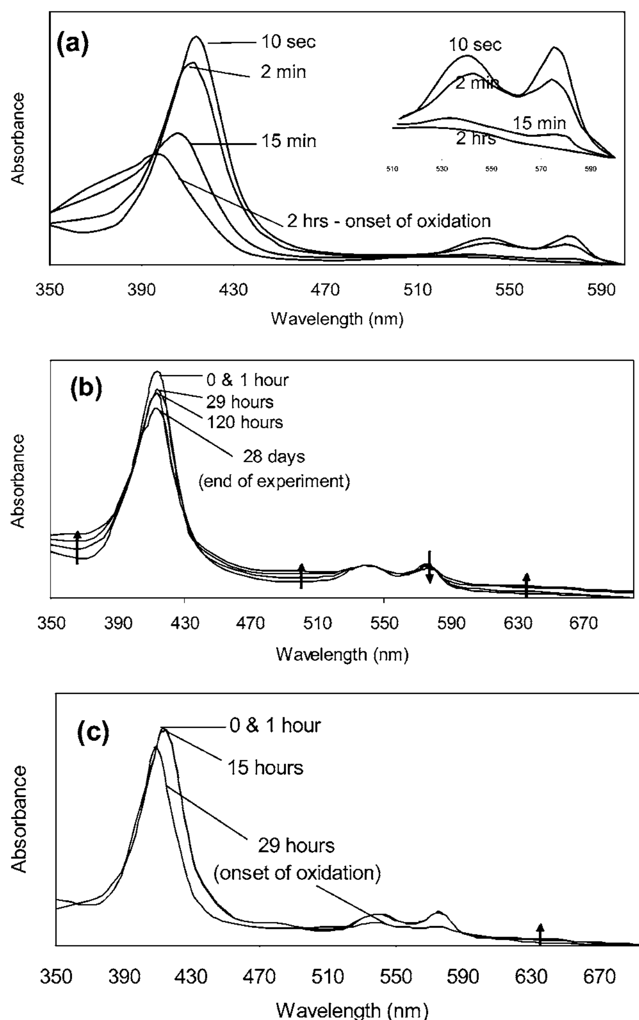

Figure 2. Changes in the visible spectra of trout hemoglobin as a function of time at pH 3 (**a**), pH 10.5 (**b**), and pH 7 (**c**). The samples were stored at 5 °C.

high pH values representative of the acidic and alkaline muscle protein isolation processes. The ability of trout hemoglobin to oxidize cod membrane phospholipids at pH 2.5−3.5, 7, 10.5, and 11 is shown in **Figure 1**. At low pH, the hemoglobin was far more pro-oxidative than at pH 7, and a value of >20 μmol (upper level of detectable rancidity by sensory) TBARS/kg tissue occurred after only ca. 3 h (**Figure 1a**). There was no significant difference ($p > 0.05$) observed for hemoglobin at pH 3 or 2.5 for the first 20 h. The pH 2.5 had, however, significantly ($p < 0.05$) higher TBARS after 20 h than pH 3. The development of oxidation at pH 3.5 was significantly ($p < 0.05$) delayed as compared to pH 2.5 and 3 reaching 20 μmol TBARS/kg tissue in ∼9 h as compared to ∼3 h for pH 2.5 and 3. Although the oxidation proceeded faster at a low pH, lower TBARS were formed as compared to pH 7. No oxidation had developed even after 28 days of refrigerated storage at pH 10.5 and 11, pointing to the high stability and inactivity of the hemoglobin under alkaline conditions. No oxidation was detected for controls without trout hemoglobin added at pH 2.5, 7, or 10.5 (data not shown).

The presence of salt was also investigated at low and high pH. This was of interest since the salt content may vary in the acidic and alkaline processes. In addition, we have previously shown that hemoglobin is less unfolded in the presence of salt at low pH (*27*). The increase in TBARS at pH 3 and 7 in the presence of 500 mM NaCl occurred somewhat later as compared to the same pH values in the absence of salt suggesting that hemoglobin was less pro-oxidative at low pH in the presence of high salt (**Figure 1b**). Hemoglobin in salt did, however, have higher TBARS ($p < 0.05$) after 20 h as compared to hemoglobin without salt, which could be due to the later onset of oxidation, and thus the later decrease in TBARS.

Changes in the conformation of hemoglobin were investigated as a function of the same time, temperature, and pH values used in the oxidation experiments. **Figure 2** shows spectra representing selected time points. At low pH (pH 3 shown), significant changes in the hemoglobin Soret peak (degradation, broadening, and blue shifting) and oxygen binding spectral peaks occurred within minutes (**Figure 2a**). At alkaline pH (pH 10.5 shown), the visible spectra were rather stable at 5 °C (**Figure 2b**). After 29 h, the heme peak (i.e., Soret peak) was slightly degraded but not shifted. Only slightly more degradation of the peak was observed on further storage. After 120 h, a small drop in the second oxygenation peak was seen at pH 10.5. Keeping hemoglobin at pH 7, on the other hand, resulted in autoxidation and deoxygenation in as little as 29 h (the onset of lipid oxidation) since the heme peak was degraded and shifted to a lower wavelength along with the drop in absorbance of the oxygen peaks (**Figure 2c**).

Pro-oxidative Activity of Hemoglobin after pH Readjustment. As the material subjected to low or high pH in the acidic/alkaline process is eventually readjusted to neutrality, it was of interest to examine the activity of hemoglobin after pH readjustment, i.e., refolding. Two holding times were employed, 90 s and 20 min, followed by readjustment of the system to pH

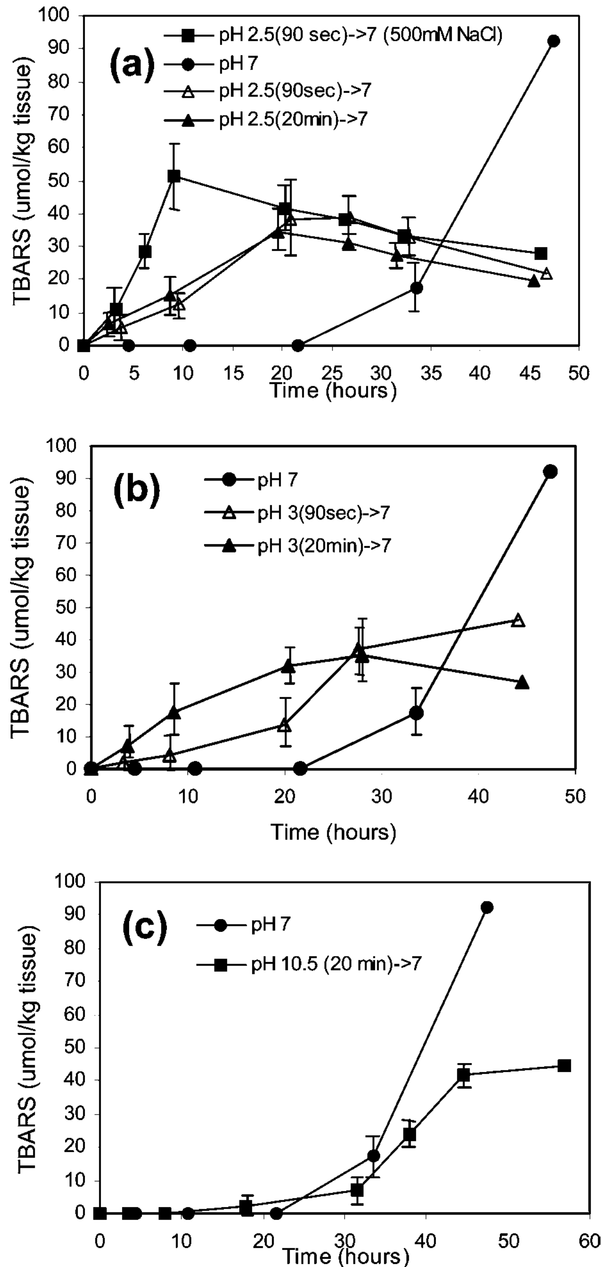

Figure 3. Effect of different holding times at acidic and alkaline pH on lipid oxidation after readjustment to pH 7. Hemoglobin and washed cod were adjusted to pH 2.5 in the absence or presence of salt (**a**), to pH 3 in the absence of salt (**b**), and to pH 10.5 in the absence of salt (**c**) and then readjusted after the time specified in the legends. The experimental conditions were identical to those in **Figure 1**.

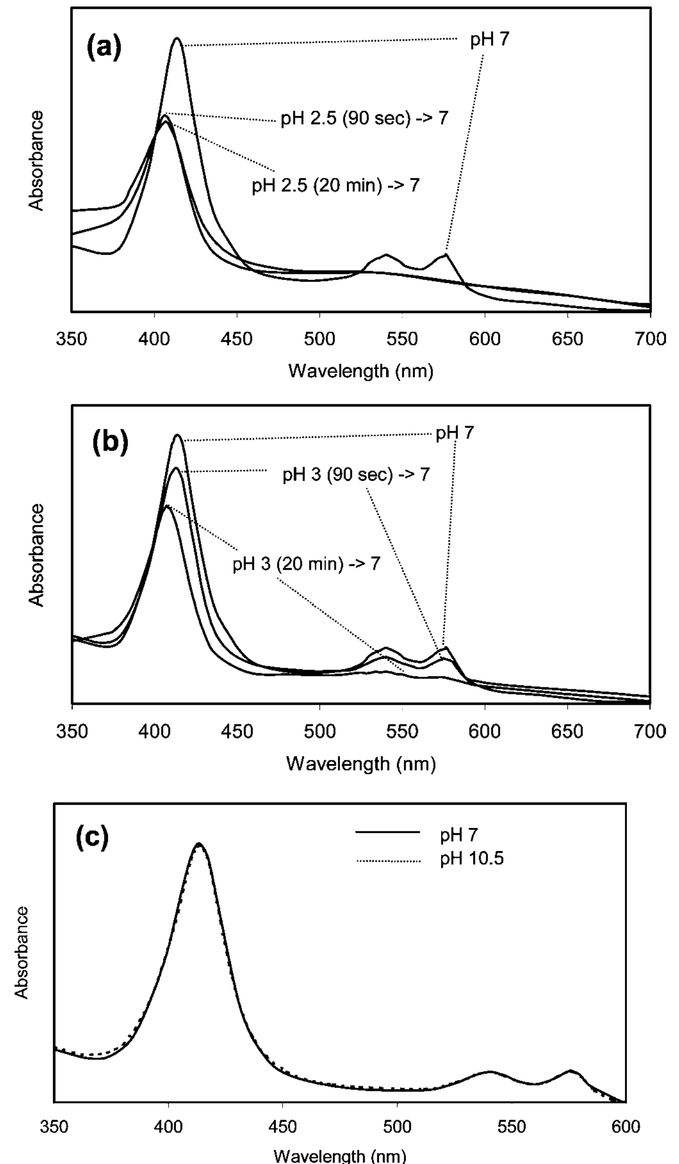

Figure 4. Spectral changes of trout hemoglobin after adjusting to pH 7 from (**a**) pH 2.5 for 90 s or 20 min, (**b**) pH 3 for 90 s or 20 min, or (**c**) pH 10.5 for 20 min. The proteins were unfolded at 5 °C.

7 and holding it at 5 °C. The results clearly show that when the mixture of washed cod muscle and hemoglobin was adjusted to a low pH followed by readjustment to neutrality, the development of TBARS was more rapid as compared to the system that was not subjected to low pH, although the amounts of TBARS were less (**Figure 3a**,**b**). Subjecting hemoglobin and the washed cod muscle to pH 2.5 prior to pH readjustment led to more pro-oxidative activity as compared to subjecting it to pH 3. Furthermore, the longer the system was held at pH 3 (i.e., 20 min vs 90 s), the more pro-oxidative hemoglobin became on pH readjustment. The holding time at pH 2.5, however, made little difference in terms of pro-oxidative activity. The presence of 500 mM NaCl during acidification and pH readjustment significantly ($p < 0.05$) enhanced the ability of the refolded species to oxidize cod membrane lipids as compared to hemoglobin in the absence of salt (**Figure 3a**). The hemoglobin readjusted from pH 10.5 had, however, no enhanced pro-oxidative activity; oxidation was in fact slightly delayed as compared to hemoglobin at pH 7, and the total oxidation was less (**Figure 3c**).

To get insight into the causes for the different oxidation development of the pH-readjusted systems, conformational changes in the protein were investigated employing the same pH treatments (**Figure 4a**−**c**). The spectra of the hemoglobin after treatment at pH 2.5 for 90 s or 20 min were similar, suggesting that changes in conformation occurred very quickly and were maintained after pH readjustment to 7 (**Figure 4a**). The hemoglobin was clearly improperly refolded after this treatment since its heme group was degraded and blue shifted and the oxygenation peaks were not recovered (**Figure 4a**). The unfolding time was more important at pH 3. A period of 90 s at pH 3 led to more recovery in the structure of hemoglobin as compared to 20 min at pH 3 (**Figure 4b**), which correlated with the less pro-oxidative activity observed (**Figure 3b**). Treating

hemoglobin to pH 10.5 for 20 min led to no obvious structural changes (**Figure 4c**).

Role of the Washed Cod Matrix vs Hemoglobin in the Oxidation. It was observed that the washed cod matrix had clearly different physical characteristics at extremes of pH, being substantially more viscous and gluelike and, on pH readjustment, had a foamlike characterstic as compared to the untreated system. For this reason, it was of interest to determine if acid- and alkali-induced changes in the washed cod muscle might have been in part responsible for some of the changes observed in TBARS production. Two experiments were performed to compare the impact of low and high pH on the washed cod muscle matrix vs hemoglobin in the lipid oxidation: (i) The washed cod was adjusted to pH 2.5 or 10.5 for 20 min and readjusted to pH 7 with untreated trout hemoglobin added at 5.8 μmol/kg tissue, and (ii) the trout hemoglobin was adjusted to pH 2.5 for 90 s or 20 min or pH 10.5 for 20 min and readjusted to pH 7 and then added (5.8 μmol/kg tissue) to untreated washed mince at pH 7.

Acidifying only the washed cod matrix and readjusting it back to pH 7 and then mixing it with native hemoglobin made it oxidize somewhat faster but produced less total TBARS untreated washed cod at pH 7 (**Figure 5a**). Interestingly, although the matrix seemed more susceptible toward oxidation after acidification, significantly ($p < 0.05$) greater oxidation (both in terms of rate and quantity) developed when acid-treated hemoglobin (pH 2.5 or 90 s and 20 min) was added to an untreated matrix at pH 7 as compared to when hemoglobin and washed cod were acidified and readjusted to neutrality together (**Figure 5b**). Interestingly, washed cod that had undergone an alkali treatment did exhibit slightly more stability at pH 7 than the untreated washed cod at pH 7 (**Figure 5a**). This was in good agreement with that observed when hemoglobin and washed cod were subjected to alkali treatment and neutral readjustment together (**Figure 3c**). Adding a hemoglobin solution that had been adjusted to pH 10.5 for 20 min and readjusted to pH 7 to untreated washed cod at pH 7 resulted in a very similar oxidation development as washed cod at pH 7 containing untreated hemoglobin (**Figure 5c**). This provides evidence that changes in the washed cod matrix may be responsible for the increased stability seen when untreated hemoglobin was added to an alkali-treated washed cod (**Figure 5a**).

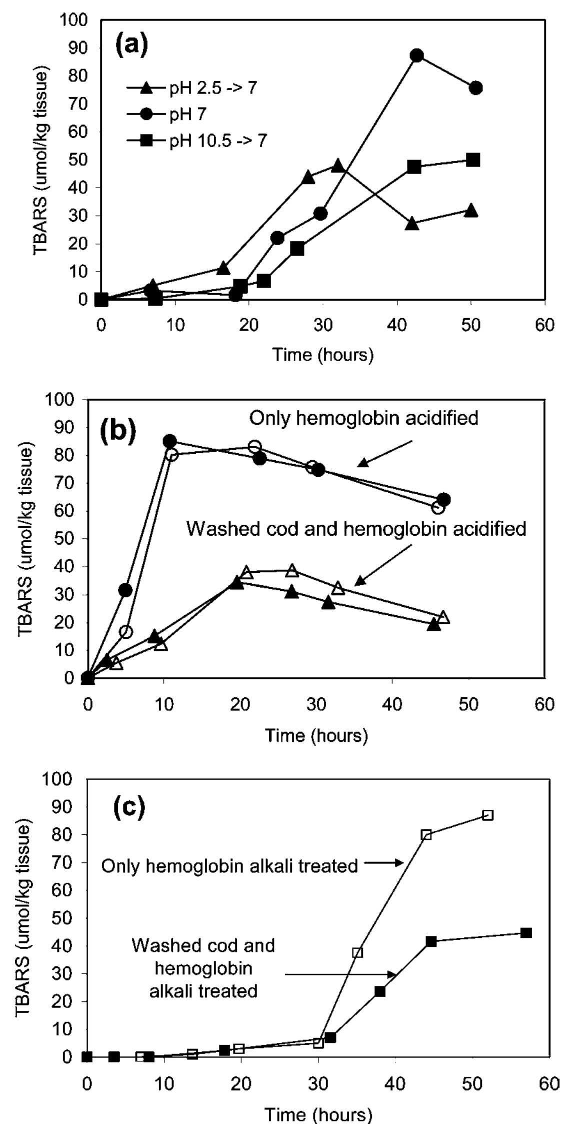

Figure 5. Role of the washed cod matrix in oxidation. (**a**) Washed cod was subjected to pH 2.5 or 10.5 for 20 min followed by readjustment to pH 7 with untreated hemoglobin added. (**b**) The hemoglobin was adjusted to pH 2.5 for 90 s (○) or 20 min (●) followed by readjustment to pH 7 and was then added to untreated washed cod at pH 7. Washed cod and hemoglobin acidified together at pH 2.5 for 90 s or 20 min and readjusted to pH 7 are included for comparison (△, 90 s; ▲, 20 min). (**c**) The hemoglobin was adjusted to pH 10.5 for 20 min and then readjusted to pH 7 and added to untreated washed cod at pH 7 (■). Washed cod and hemoglobin treated to an alkaline pH (pH 10.5 for 20 min) and readjusted to pH 7 are included for comparison (□). The experimental conditions were identical to those in **Figure 1**.

DISCUSSION

Lipid oxidation is a major cause of quality deterioration in muscle foods, and hemoglobin can be the main pro-oxidant under certain conditions (*2*, *4*). The presence and functionality of hemoglobin are of great interest to an acidic and alkaline muscle protein solubilization process aimed at producing functional protein isolates from underutilized sources of fish. This is because the raw material for the process is often rich in heme proteins, and hemoglobin may be present in sufficient amounts in the final product to cause oxidative and color problems. It was therefore of interest and importance to investigate how low and high pH conditions would affect the pro-oxidative ability of hemoglobin and also how it would behave when readjusted back to neutral pH values.

Trout Hemoglobin Has Greatly Enhanced Pro-oxidative Activity at Low pH. The hemoglobin became significantly more pro-oxidative at low pH vs neutral and alkaline pH (**Figure 1**). There are many possible reasons for this increased activity at low pH. In solution at low pH, it was evident from the nature of the hemoglobin visible spectra from 350 to 700 nm that the protein unfolded and that its heme iron oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form. The formation of the oxidized forms, methemoglobin and metmyoglobin, is known to occur more rapidly as the pH is lowered (*11*). This proton dependency is believed by some to be mediated via the distal histidine in hemoglobin, which normally forms a hydrogen bond to the O_2 molecule (*8*). At lower pH, the distal histidine apparently facilitates the movement of a proton from the solvent to the bound polarized O_2 by a proton relay mechanism (*11*, *28*). The rate constant for this mechanism increases as more protons become available, i.e., as the pH is lowered. The significant blue shift and broadening of the heme peak (i.e., Soret peak) at a low pH are indications that the interaction of the heme and

the distal histidine is lost (*29*). Peak broadening can also be in part attributed to the loss of the O_2 ligand. Once oxidized, the protein can be activated by preformed hydroperoxides in the washed cod muscle and transformed into a high valency (Fe^{4+}) ferryl species, a reaction that is favored as the pH is lowered (*30*). This species is widely believed to be the initiator of many lipid peroxidation reactions in foods and living tissue (*7*). Superoxide ($O_2^{\bullet -}$), which is released from hemoglobin on autoxidation, forms the thermodynamically more reactive and unstable hydroperoxyl radical ($HOO^{\bullet}$) at a low pH (pK_a of 4.8) (*19*, *31*). This radical has the ability to penetrate into the hydrophobic lipid region of the membrane bilayer, making it an effective pro-oxidant (*19*). It could be one of the causes of the rapid lipid oxidation observed with trout hemoglobin at a low pH. In addition to the above, we have previously found that trout hemoglobin dissociates into a mixture of monomers and tetramers at low pH (*27*). Monomers are known to autoxidize faster than tetramers (*11*, *12*, *15*) and be more prooxidative (*16*), which could in part explain the increased activity at low pH. Dissociated subunits are also expected to increase the accessibility of hemoglobin to lipid substrates as they could be better distributed in the system.

The reason for the rapid oxidation at low pH may not only be explained by enhanced autoxidation and favorable conditions for radical formation. The substantial conformational changes in the protein global structure and heme crevice likely play a role in the increased activity of trout hemoglobin. Hemoglobin is a highly water soluble protein with relatively few hydrophobic groups exposed in its native state. However, on acid unfolding, the protein does expose hydrophobic clusters otherwise buried (*27*, *32*). We have previously reported that hemoglobin is in a molten globular state at low pH (*27*, *32*). This means that the protein has intermediate characteristics of a folded and unfolded protein, being more structurally flexible and unstable. It is known that some proteins in the molten globule state can more readily interact, penetrate into, and transfer across membranes due to their hydrophobic and flexible nature (*33*). Trout hemoglobin at acid pH may therefore interact more strongly with the cod membrane lipids as compared to pH 7 as it is more flexible, open, and hydrophobic and could perhaps even penetrate into the membrane bilayer thus exerting its action more effectively. Because of the significantly exposed heme group at low pH, it is possible that fatty acids would have easier access to the crevice (*21*). The spectra of hemoglobin also indicate that the heme group may lose its contacts with histidine at low pH since it is significantly blue shifted (*27*, *32*). This would make the now exposed highly hydrophobic heme very susceptible to partition into a nonpolar phase rather than being exposed to solvent (*20*). The possibility therefore exists that at low pH trout hemoglobin may lose its heme to the membrane phospholipids as it is no longer stabilized to the same extent in the globin and has high affinity for nonpolar phases. If heme would be lost to the membrane, lipid oxidation would be expected to occur much faster than when it is associated with the globin since it greatly moderates the action of heme. Eriksson and coworkers (*34*) found free heme to be far more pro-oxidative on linoleic acid than when intact in catalase or peroxidase. A number of studies have shown that free heme can rapidly dissolve into the membrane bilayer and can easily translocate across membranes and as a result lead to more oxidative and structural damage of lipid membranes than when it is in the globin (*16*, *22*, *35*).

The increased hemoglobin pro-oxidative activity at low pH is most likely a complex combination of the above scenarios and needs further detailed study. The differences seen with pH 2.5, 3, and 3.5 in oxidation rates could be explained by differences in conformation. The protein at pH 3.5 is less unfolded with its heme group less exposed and is less hydrophobic than at pH 3 or 2.5 (*32*). This may explain why hemoglobin at pH 3.5 is not as active as the protein at pH 3 and 2.5. The different molten globule intermediates may thus have different pro-oxidative activities modulated by their different conformations. It was interesting to note, however, that although low pH led to a more rapid oxidation the maximum level of TBARS produced was significantly ($p < 0.05$) lower than the maximum level of TBARS produced at neutral pH. The same was seen after refolding and is discussed later. The mechanism behind this is not known, but many possibilities would exist in a complex system like this one. It is possible that the low pH causes a physical change in the membrane structure, which would lead to less formation of oxidation products. If membranes aggregate, there would be less substrate (due to less accessible surface area) for the hemoglobin and the level of oxidation would be expected to be less, although the onset of the reaction would be sooner than at pH 7.

An increased ionic strength moderates the activity of trout hemoglobin at low and neutral pH. Salt may occur at varying levels in the acidic and alkaline process and is normally included in a final product, such as a fish muscle gel. Sodium chloride has been found by some workers to lead to more rapid oxidation in muscle systems (*36*) while others report an inhibitory effect (*37*). In the presence of 500 mM NaCl, trout hemoglobin was less pro-oxidative than in its absence at both low and neutral pH. This may be due to the salt's action of lowering the electrostatic attraction between the hemoglobin and the membrane, as salt could screen the charges. It is not straightforward how electrostatic attractions in this complex system could play a role at the low pH values used in this experiment (pH 2.5–3.5). However, if the phospholipid retains its negative charge at low pH, it may attract the positively charged hemoglobin, and the presence of salt may lower this attraction. At this pH in salt, hemoglobin was also found to be considerably less unfolded as compared to the same pH in the absence of salt, and the Soret spectrum was different (*27*). The conformational data indicated that the heme crevice was less unfolded and the heme group may have been less exposed to the solvent as compared to pH 2.5 in the absence of salt. This could also explain the lower pro-oxidative activity.

Alkaline pH Inhibits the Pro-oxidative Activity of Trout Hemoglobin. Oxidation was dramatically inhibited at alkaline pH, with almost no development in TBARS even after 28 days (the time at which the experiment was stopped) (**Figure 1**). At the same storage time and temperature in solution, trout hemoglobin changed little in structure and was stable toward autoxidation, as seen from its visible spectra (**Figure 2c**). As the oxygen peaks between 500 and 600 nm in the visible spectrum of trout hemoglobin change little with time at alkaline pH, it is evident that O_2 is strongly associated with the hemoglobin. This resistance toward autoxidation and inactivity of hemoglobin at alkaline pH is very likely attributed to the strong bond between the distal histidine in hemoglobin and the O_2 since the distal histidine serves as a barrier against autoxidation (*38*). The reactivity toward preformed hydroperoxides therefore is reduced, and as the H^+ concentration is extremely low at this pH, H^+ is unable to lead to autoxidation via nucleophilic displacement of oxygen. Alkaline pH has been shown to stabilize heme proteins, but under strong alkaline conditions (above pH 10), autoxidation increased and most

proteins were converted to the met form within several hours or days (*11*). No significant autoxidation was seen with trout hemoglobin at pH 10.5 and 11 in this study. As hemoglobin did not oxidize, the conformation of the heme pocket would be expected to be largely intact at pH 10.5. The slight decrease, however, seen in the heme peak at pH 10.5, but not blue shift, with time indicates that the heme may become hexa-coordinated (*39*), possibly with the distal histidine as it may lose part of its contact with O_2 on prolonged storage. There is in fact a very small drop in one of the subunit oxygenation peaks seen with time (**Figure 2b**). This hexa-coordination and drop in heme peak have been found to occur at alkaline pH in peroxidases (which have a very similar heme environment as hemoglobin) and caused them to lose their peroxidase activity (*39*, *40*). Trout hemoglobin is therefore likely inactivated at high pH, which could prevent the heme from participating in lipid oxidation reactions since it would be protected from access to lipids or dissociation into the lipid bilayer of membranes.

It is worth keeping in mind the possibility that muscle protein sulfhydryl groups in the washed cod at alkaline pH may participate as antioxidants, as they become very reactive at high pH. The antioxidant activity of human serum albumin increased steeply as the pH was shifted from neutral to alkaline pH due to the increased participation of sulfhydryl groups (*41*).

Hemoglobin Has Increased Pro-oxidative Activity after pH Readjustment from Low pH. As the final material in the acid and alkali muscle protein isolation processes will eventually be brought from acidic or alkaline pH to pH values around neutrality, it was important to determine how pro-oxidative activity and protein structure are changed on pH readjustment. It was found that the lower the pH (2.5 vs 3) and the longer the holding time (90 s vs 20 min) at low pH, the more misfolded the protein was and in turn the more pro-oxidative the hemoglobin became. It is tempting to attribute this enhanced activity to protein misfolding, as the more pro-oxidative protein represented those that had less recovery in native structure. We have previously reported that the lower solubility of misfolded hemoglobin was related to increased hydrophobicity (*27*), which may suggest that on refolding the protein can more easily associate with the lipids in the cell membranes of the washed cod and also the other misfolded proteins in the system. At pH 2.5, a holding time of 90 s or 20 min made little difference in native structure recovery as judged by the visible spectrum of hemoglobin (**Figure 4a**), possibly because after 90 s the protein had apparently unfolded to the extent it could at that pH (*27*). As demonstrated in **Figure 3**, the pro-oxidative activity of the refolded hemoglobin was almost identical regardless of whether it was held for 90 s or 20 min at pH 2.5. After 90 s at pH 3, trout hemoglobin only unfolds to a small extent as compared to 20 min (*27*), and recovery in the native structure is therefore easier to accomplish (**Figure 4b**). This small change in conformation at pH 3 for 90 s as compared to 20 min correlated with less pro-oxidative activity. These results suggest that changes in conformation are the cause for the different pro-oxidative activities. Although much structure is retained after 90 s at pH 3, the protein was still significantly more pro-oxidative than native hemoglobin. It is a possible that hemoglobin may have refolded differently in-situ (i.e., in the system) as compared to the solution used for conformational determination, which could aid in explaining the strong pro-oxidative activity.

When trout hemoglobin and the washed muscle were acidified (pH 2.5) and readjusted to neutrality together in the presence of 500 mM NaCl, the hemoglobin was found to be more pro-oxidative than in the absence of added NaCl, even though it was found to be less pro-oxidative at pH 2.5 in 500 mM NaCl. We have previously demonstrated that refolding hemoglobin from low pH to pH 7 in salt as compared to no salt led to less recovery in native structure (*27*). This suggests that adverse conformational changes play a role in the increased activity. When proteins were refolded from pH 2.5 to 7, they were also found to be more prone to aggregate via hydrophobic interactions (*27*). This could make the protein interact with the lipids in the membranes more effectively and perhaps more readily give up its heme than the hemoglobin refolded in the absence of salt.

There was no study found in the literature that has addressed the pro-oxidative activity of refolded heme proteins. However, Erickson and co-workers (*34*) did report that the pro-oxidative activity was higher for recombined heme and peroxidase (apo form) as compared to the native peroxidase but did not investigate the conformation of the protein. It has been reported by others that the recombination of free heme to apohemoglobin leads to only partial recovery in structure (*20*) with the heme group possibly more exposed in the recombined complex as compared to the native complex.

Potential Role of the Washed Cod vs Hemoglobin in the Lipid Oxidation. It is worth mentioning that not only changes in the protein conformation but also structural changes or reorganization of the lipid bilayer can be responsible for the ability of hemoglobin to interact with the membrane in the washed cod system and oxidize its lipids. The dramatic changes that occur with the muscle cell at low and high pH break up the cell structure since the muscle proteins are solubilized and can be partly separated from the membrane lipids (*19*). It is unknown what effect low or high pH has on the cod muscle membrane structure. It would likely involve considerable structural changes since the membrane phospholipids are charged molecules. It was therefore important to know if in fact conformational changes in hemoglobin, membrane structural changes, or both were the primary cause of the oxidation.

By adding acidic unfolded and refolded hemoglobin (90 s and 20 min at pH 2.5 and then adjusted to pH 7) to untreated washed cod muscle and comparing it to a system where both hemoglobin and washed cod are acid-treated and readjusted, one can get insight if the rapid oxidation seen for the acidified and readjusted hemoglobin and washed cod muscle was due to the effects of pH on the hemoglobin or on the washed cod muscle or both (**Figure 5b**). That the acidic unfolded and refolded hemoglobin caused a significantly more rapid oxidation on the untreated substrate and higher TBARS values was strong evidence that the misfolded hemoglobin structure rather than changes in the washed cod muscle is more important in the oxidation development. The lower pro-oxidative activity of the trout hemoglobin acid-treated with the washed cod muscle, as compared to when it is added to a untreated muscle, may be attributed to physical changes of washed cod muscle and/or the interaction of refolded hemoglobin with components other than lipids.

The role of the washed cod muscle was further assessed by subjecting it to low or high pH and then readjusting it to pH 7 and adding untreated native hemoglobin to it. This should put the acidic, alkaline, and untreated washed cod muscle on an equal level of comparison, and oxidation development should be identical if changes in washed cod muscle were of no consequence to oxidation. This is clearly not the case if one looks at **Figure 5a**. That acid-treated washed cod muscle oxidizes fastest points to a change in the substrate that makes

the membrane lipids more susceptible to oxidation. Trout hemoglobin may thus have easier access to the lipids of the washed cod mince membranes due to the membranes disorganization and possibly more open structure after acid treatment. Even though the washed cod muscle readjusted from low pH oxidized faster, it developed lower TBARS values as compared to untreated washed cod muscle, which suggested it is due to changes in the washed cod on acid treatment. These changes appeared to remain after pH readjustment, as discussed above, suggesting that the change may be permanent. The molecular mechanisms behind these changes are currently not known but are under investigation. There are many possible explanations for this in such a complex system. The possibility exists that hemoglobin may have had less access to the lipids in pH-treated washed cod muscle. Hemoglobin may have become coaggregated with the muscle proteins when they were neutralized after acid treatment, thus lowering the contact with lipids. It is also possible that the physical changes occurring with the membranes at low pH may have led to membrane aggregation, thus decreasing their surface area and in turn making them less susceptible to oxidation. The alkali-treated washed cod muscle in contrast to the acid-treated muscle appeared to be less susceptible toward oxidation at pH 7 as compared to untreated washed cod muscle, also developing lower TBARS values like the system after acid treatment. As phospholipids are charged molecules, the membrane may react differently to acidic and alkaline pH, yielding different structures on refolding. For example, erythrocyte cell membranes lose their structure and lyse when incubated at acidic pH (pH 2−5) (*42*). Alkali-induced breaking of erythrocyte membranes is, however, reported to be less than acid-induced breaking (*43*), which could contribute to the lower susceptibility of the alkali-treated washed cod muscle. Another possibility for the increased stability after alkaline treatment may be due to more reactive (i.e., exposed) sulfhydryl groups on the muscle proteins that are a large part of the washed cod muscle. Sulfhydryl groups of proteins are competitively oxidized at the expense of the lipids and thus delay oxidation (*41*). This role of sulfhydryl groups has been demonstrated by Soyer and Hultin (*44*) with isolated cod sarcoplasmic reticulum, which is the main source of membrane lipids in the current study. It remains to be determined whether the increased oxidative stability of the alkali-treated system is due to more available reactive sulfhydryls. The changes brought about in the substrate will be important to understand for the acidic and alkaline processes and deserve future study.

In conclusion, this study demonstrated that trout hemoglobin becomes highly pro-oxidative at very low pH values (pH 2.5−3.5) and also after pH readjustment to neutral pH. This enhancement in activity is believed to be due to significant conformational changes occurring in the protein at acidic pH values and lack of refolding on pH readjustment. A highly alkaline pH (pH 10.5−11), on the other hand, significantly retarded the pro-oxidative activity of hemoglobin, possibly due to stabilization of its protein structure and oxidative state. This study also demonstrated that pH treatment influences the susceptibility of the washed cod substrate to oxidize, leading to less formation of lipid oxidation products than an untreated system. This study clearly indicates that lipid oxidation could become a significant problem in fish protein recovery processes using low pH values if raw material is rich in heme proteins. Processing at high pH, on the other hand, appears to have a stabilizing effect with respect to lipid oxidation and would be highly favored over low pH.

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